

# SLAMF6 in Health and Disease: Implications for Therapeutic Targeting

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## 1 Introduction

Effective immune responses develop after a well-orchestrated series of events that include recognition, immune cell interactions and activation/inhibition of signaling pathways. The signaling lymphocyte activation molecule family (SLAMF) of cell surface receptors, which consists of nine transmembrane proteins (SLAMF1-9) expressed at different levels, are involved in viral and bacterial recognition, serve as co-stimulatory molecules at immune synapses, and modulate myeloid and lymphocyte development. SLAMF receptors are homophilic receptors, with the exception of SLAMF2 and SLAMF4, and are only expressed on hematopoietic cells. Their adaptors, SLAM associated protein (SAP) and Ewing's sarcoma-associated transcript 2 (EAT-2), bind to the cytoplasmic tails and control the functions and magnitude of SLAMF receptor signaling. In this review, we focus on the current knowledge on the role of SLAMF6 receptor in regulating immune functions and recent findings describing how SLAMF6 can be exploited as a target in human malignancies.

## 2 A Background of the SLAM Family Members and adaptors SAP and EAT-2

### 2.1 Structure

The SLAM family of immune cell surface receptors is a member of the CD2 subfamily of the immunoglobulin (Ig) superfamily consisting of nine members, SLAMF1-9 [1-4]. SLAMF receptors are type I transmembrane glycoproteins comprised of an extracellular membrane containing an N-terminal V-Ig domain followed by a C2-Ig domain in the extracellular region (this set is duplicated in SLAMF3), a transmembrane region, and an intracellular cytoplasmic tail containing tyrosine based switch motifs (ITSM). Notable exceptions to this structure include SLAMF2, which has a glycosyl-phosphatidyl-inositol (GPI) membrane anchor and like SLAMF8 and SLAMF9 lack ITSM motifs [5-8]. Binding of SLAM associated adaptors; SAP and EAT-2, to cytoplasmic tails of various SLAMFs regulate their function on different immune cells. Expression of SLAMFs and their adaptors is restricted to hematopoietic cells. In addition, the gene loci are located on chromosome 1 in both mice and humans, except SAP, which is located on the X chromosome [9](**Figure 1**).

All SLAMFs are homophilic receptors aside from SLAMF2 and SLAMF4, which bind each other [10-12]. The determination of the SLAMF3, SLAMF5 and SLAMF6 crystal structures revealed *in-trans* interactions through their IgV domains (SLAMF3 unpublished data, generously donated by Profs. Steve Almo and Stanley Nathenson, Albert Einstein College of Medicine (**Figure 2**) [13, 14]. Engagement of SLAMF

receptors on immune cells (e.g. APC - T cell) trigger inhibitory or activating signals that modulate cellular responses. Within these homophilic and heterophilic interactions, the binding affinities for each SLAMF varies (SLAMF3 nM, SLAMF5 sub- $\mu$ M, SLAMF6 ~ 2  $\mu$ M, SLAMF2/4 ~4  $\mu$ M, SLAMF1 ~200  $\mu$ M) which likely contributes to functional differences within the family of receptors [12-15]. In addition to being self-ligands, SLAMF1 also serves as an entry receptor for Measles virus [16, 17] while SLAMF1, SLAMF2 and SLAMF6 have been demonstrated to interact with bacterial components [18-21] (reviewed in detail ref [22]).

## 2.2 SAP and X-linked Lymphoproliferative Disease (XLP)

First identified in 1975, X-linked lymphoproliferative disease (XLP) (also known as Duncan's disease) is an extremely rare primary immunodeficiency that mainly manifests in males and is primarily characterized by extreme susceptibility to infection with Epstein-Barr virus (EBV) [23]. However, most XLP patients infected with EBV develop fatal or fulminant infectious mononucleosis due to dysregulated immune responses, which leads to clonal proliferation and expansion of T and B cells. NK and CD8<sup>+</sup> T cell functional defects have been identified in XLP patients likely contributing to the inability to control EBV infections [24-27]. Patients usually exhibit progressive loss of serum IgG and develop B cell lymphomas and dys-gammaglobulinemia [28-31].

Not until twenty years after the description of XLP, the genetic cause was determined by the Terhorst lab: mutations in or deletion of the *SH2D1A* gene, which encodes a 15 kD cytoplasmic protein SAP consisting of a single Src homology 2 (SH2) domain and a 28

amino acid tail [32-35]. In the same publication, SAP was shown to bind to SLAMF1 and subsequent studies showed binding of SAP to ITSM motifs in the cytoplasmic tail of six of the SLAMF receptors (**Figure 3**) [1, 30, 34, 35]. SAP was then identified to be required for recruitment and activation of Src-family kinase FynT upon SLAM ligation [36]. Subsequently, the crystal structure of the SLAM-SAP-Fyn-SH3 ternary complex revealed that SAP binds the FynT SH3 domain through a non-canonical surface interaction and couples Fyn to SLAM receptors (**Figure 4**) [37].

SAP is mainly expressed in T cells, NK cells, NKT cells and eosinophils while expression in B cells is found only in some cases [33, 34, 38-40]. Of note, later research also identified mutations in *XIAP*, X-linked inhibitor of apoptosis, which are associated with XLP like disease manifestations in a small number of families [41, 42].

Studies from SAP deficient mice shed light on understanding the basis of XLP. Naïve CD4<sup>+</sup> T cells from SAP deficient mice exhibited reduced production of peptide-MHC or T cell receptor (TCR) driven T<sub>H</sub>2 cytokines [43-46]. In addition, germinal center formation is significantly impaired and antibody secreting cells (ASCs) and memory B cells are lost leading to severely reduced levels of serum IgG and IgE [43, 47-49]. Later, these defects were also identified in XLP patients [50, 51].

In SAP deficient mice there appeared to be a B cell defect, which was dependent on the genetic background [52] as evidenced by adoptive transfer studies of SAP deficient B cells and WT CD4<sup>+</sup> T cells [47]. Although the B cell defect seems secondary to that of CD4<sup>+</sup> T cells [48, 49, 51], *in vitro* experiments suggested an intrinsic defect in class switching [40].

CD8<sup>+</sup> T cells and NK cell functions are also altered in SAP deficient mice and XLP patients. While human *in vitro* studies suggested SAP is required for induction of cytotoxicity by CD8<sup>+</sup> T cells [53], SAP deficient mice presented more virus specific CD8<sup>+</sup> T cells in lymphocytic choriomeningitis virus (LCMV) and  $\gamma$ -herpesvirus 68 infections, suggesting SAP inhibits CD8<sup>+</sup> T cell responses [54, 55]. Finally, SAP was found to be indispensable for NKT cell development as SAP deficient mice and XLP patients lack NKT cells [56-58].

In summary, SAP signaling is critical for mounting proper immune responses. Moreover, SAP, as well as EAT-2, regulates signaling through SLAMFs by setting thresholds for activation/inhibition and modulating cell-cell interactions and responses. Here, we will highlight the roles of SLAMF receptors in regulating normal immune responses and how they are involved in hematological malignancies.

### 3 Immune cell Functions of SLAMF6 (CD352, Ly108, NTB-A)

SLAMF receptors are adhesion molecules that are involved in development of lymphocytes and in orchestrating innate and adaptive immune responses. While contribution of each SLAMF member is unique, they can have compensating or opposing roles in function. Role of SLAMF6 in immune regulation will be discussed in this section.

#### 3.1 Structure, expression and ligands

SLAMF6 (human: NTB-A, mouse: Ly108) structural details are reviewed elsewhere [3]. In mice, different alternatively spliced forms exist: Ly108.1 and Ly108.2 were first identified, containing one and two additional unique tyrosine motifs, respectively [59, 60]. Later a novel isoform Ly108-H1 was discovered [61]. Tyrosine phosphorylation of the cytoplasmic tail of SLAMF6 leads to recruitment of SAP with high affinity binding, but in the absence of phosphorylation, SAP cannot be recruited [3, 62, 63]. Binding of SAP activates downstream signaling by recruiting the Src family kinase Fyn [37, 64, 65]. This interaction prevents the tyrosine phosphatases SHP1 and/or SHP2 from binding to the cytoplasmic tail and their subsequent negative regulation [34, 35].

SLAMF6 is expressed on a wide variety of immune cells including T cells (also T<sub>FH</sub>), B cells, NK cells (expressed in human only), double positive thymocytes, eosinophils and neutrophils (mouse only) [6, 38, 59, 66-69]. Furthermore, high expression of SLAMF6 has been determined in various B cell lymphomas, *i.e.* mantle cell and follicular

lymphomas [70]. Expression on normal as well as malignant cells suggests that SLAMF6 may be involved in distinct cell-cell interactions in different microenvironments and may be a useful therapeutic target.

A SLAMF6 homolog, S1, with a 97% amino acid sequence identity in its ligand binding N-terminal Ig domain have been identified in SMCMV and OMCMV [71]. This was acquired by retrotranscription of virus-host coevolution by the new world monkey CMV. This suggests an immune evasion mechanism of viruses by acquiring the host SLAMF receptors that retain the ligand binding capacity. This allows the interference with host SLAMF functions and induces immunomodulatory actions.

In addition to being a self-ligand, SLAMF6 detects viral and bacterial components as well. For instance, human and mouse SLAMF6 bind the outer membrane proteins OmpC and OmpF, of *E.coli*, *S. typhimurium*, and in part to *Citrobacter rodentium* [18, 21]. Human SLAMF6 can recognize the influenza haemagglutinin (HA) and Vpu protein of HIV-1, both of which affect NK cell cytotoxicity mediated by SLAMF6 [72, 73].

### 3.2 SLAMF6 signaling in T and B cells

Co-stimulatory molecules play an important role in activation and initiation of proper T cell responses upon TCR engagement. CD28 is the best-known secondary signal necessary for T cell activation. However, studies from CD28 deficient mice suggested additional co-stimulatory molecules play a role as antigen dependent T cell responses were still intact in these mice [74, 75]. In the absence of CD28, stimulation and crosslinking of SLAMF6 with monoclonal antibodies *in vitro* leads to recruitment of SAP to the phosphorylated cytoplasmic tail, and subsequent events lead to T cell proliferation



and cytokine production [76]. When SLAMF6 was blocked *in vivo* using a soluble SLAMF6-Fc fusion protein, B cell isotype switching to IgG2a and IgG3 was blocked [76]. In fact, injections of anti-SLAMF6 to WT mice immunized with NP-OVA or in a chronic graft versus host disease (cGVHD) model inhibited T and B cell responses in these distinct *in vivo* models [77-79].

Involvement of SLAMF6 in regulating immune responses was demonstrated using mice deficient in SLAMF6 [80]. Expression of the SLAMF6 extracellular domain was disturbed by removal of exons 2 and 3 (Ly108  $\Delta$ E2+3) [80]. *In vivo* infection of Ly108  $\Delta$ E2+3 mice with *L. mexicana* led to delayed formation of lesions as well as significantly smaller lesions compared to WT controls, indicating a role for Ly108 in innate and adaptive immune responses.

Under normal circumstances, T cell expansion upon infections is constrained by a mechanism called restimulation-induced cell death (RICD), which induces apoptosis of effector T cells during the peak of an immune response [81]. Upon restimulation of these T cells, pro-apoptotic molecules are upregulated, such as FAS ligand and BIM, to induce apoptosis. This mechanism was found to be defective in patients with XLP, mainly due to absence of SAP and defective signaling through SLAMF6 [82]. In RICD, SAP/SLAMF6 signaling was shown to augment TCR signaling to achieve the threshold necessary for RICD. This was facilitated by SAP's recruitment of Lck, but not Fyn, to the cytoplasmic tail [83].

SLAMF6 is also implicated in trogocytosis, the transfer of membrane patches from target to effector T cells [84, 85]. The trogocytosis capacity of CD8<sup>+</sup> T cell clones from melanoma patients correlated with their cytotoxic capacity. Furthermore, higher cytotoxic capacity was correlated with increased phosphorylation of SLAMF6 [84]. Enhanced

cytotoxicity of these CD8<sup>+</sup> T cells could be blocked by an antagonist  $\alpha$ SLAMF6, indicating that SLAMF6 co-stimulation plays an active role in T cell functional diversity. As CD8<sup>+</sup> T cells from XLP patients are defective in cytolytic activity against EBV-infected B cell targets, SLAMF6 as well as SAP may be involved [53, 86-88]. Mice deficient in SAP presented altered T cell responses including short-lived T-B cell interactions, defective germinal center formation, and humoral immunity [2, 63, 89]. As CD8<sup>+</sup> T cells are critical in controlling infections, the role of SAP as well as contributions of SLAMFs were investigated. Studies using SAP deficient mouse T cells showed inefficient killing of B cell target cells, but not others, suggesting SAP is especially critical in T-B cell interactions [90]. These defects were at the level of immune synapse organization and inefficient actin clearance. Conjugation of OVA-pulsed B cells with WT or SAP<sup>-/-</sup> cytotoxic CD8<sup>+</sup> T lymphocytes (CTL) showed significant SHP-1 localization at the immune synapse with SAP<sup>-/-</sup> CTL compared to WT CTL as quantified from immunofluorescence microscopy images [90]. This suggested that in the absence of SAP, SHP1 is recruited to the cytoplasmic tail of SLAMF6 [79]. When B cell targets were SLAMF6 deficient, killing ability of SAP<sup>-/-</sup> T cells was rescued demonstrating that SLAMF6 is an important regulator of SAP dependent responses from CTLs. Our current understanding of how SLAMF6 may play a role in T-B cell interactions is summarized in **Figure 5.**

### 3.3 SLAMF6 controls neutrophil functions

Disruption of Ly108 expression in mice (Ly108  $\Delta$ E2+3) also revealed a role for Ly108 in neutrophil functions [80]. In response to infections with *Salmonella typhimurium*, neutrophils exhibited significantly reduced reactive oxygen species (ROS) production

and bacterial killing with increased IL-6, IL-12 and TNF- $\alpha$  production compared to WT controls. Mechanisms of Ly108 signaling involved in ROS production is still not known. While neutrophils are not implicated in XLP and human neutrophils do not appear to express SLAMF6, it may be of interest to investigate whether the expression can be induced upon activation.

### **3.4 SLAMF6 is an activating receptor on human NK cells**

Activating signals induced upon ligation of NTB-A on an NK cell induces phosphorylation of its cytoplasmic tail, which is Src kinase dependent, and leads to recruitment of SAP, EAT-2 and SHP1/2 [91, 92]. Full SLAMF6 dependent activation of NK cell cytotoxicity depends on simultaneous binding of SAP and EAT-2 to the phosphorylated tyrosine residues on the cytoplasmic tail [92]. Crosslinking of human SLAMF6 with antibodies stimulate NK cells for target cell killing in cytotoxicity assays, and homophilic interaction of ligands induces NK cell cytotoxicity against target cells with subsequent IFN $\gamma$  and TNF $\alpha$  secretion [66, 91]. However, if the SLAMF6 homophilic interaction between an NK cell and a target cell is blocked using an anti-SLAMF6 antibody, NK cell cytotoxicity is inhibited [69]. Interestingly, blocking the homophilic interaction between neighboring NK cells has no effect on proliferation or cytotoxicity [93]. Instead, homophilic interaction with a MHC-I negative target cell induces potent cytotoxicity of NK cells. This selective mechanism is probably in place to ensure NK cells do not end up killing each other.

While expression of SLAMF receptors is restricted to hematopoietic cells, a recent study demonstrated that SLAMF6 enhanced activation of NK cells against a non-hematopoietic target cell [94]. This was regulated by SAP, which uncoupled SHP-1 binding from the cytoplasmic tail of SLAMF6, diminishing the effect of the receptor on NK

cell responsiveness to non-hematopoietic cells. This proved that SLAM-SAP pathways could also influence NK cell education [94].

### 3.5 SLAMF6 regulates NKT cell development

Natural killer T (NKT) cells represent a subset of T lymphocytes that develop from double positive (DP) CD4<sup>+</sup>CD8<sup>+</sup> precursor cells in the thymus [95]. While positive selection of conventional T cells is mediated by interactions with thymic epithelial cells, NKT cells are selected by lipid antigens presented by CD1d on other DP thymocytes [96, 97]. These cells express an invariant TCR and can rapidly secrete cytokines following infections [98].

With the severe lack of NKT cells in XLP patients, SAP was hypothesized to be involved in NKT cell development [1]. Indeed, mouse studies showed that SAP expression in NKT cells is necessary for cognate help to B cells[99]. As binding of SAP and recruitment of Fyn is induced by signaling through SLAMF receptors, SLAMF1 and SLAMF6, which are expressed on DP thymocytes, their possible role in NKT cell development was investigated. Mouse studies from single knockouts (SLAMF1<sup>-/-</sup>, SLAMF6<sup>-/-</sup>) revealed only a modest effect on NKT cell development, probably due to overlapping functions of these two receptors [80, 100]. Supporting this hypothesis, mice reconstituted with double mutant (SLAMF1<sup>-/-</sup>SLAMF6<sup>-/-</sup>) bone marrow chimeras had severely impaired NKT cell development [68]. Furthermore, expression of promyelocytic zinc finger (PLZF), a transcription factor required for development of invariant NKT (iNKT) and other innate like T lymphocytes, was demonstrated to be modulated by co-stimulation through

SLAMF6 [101-103]. These studies together underline the importance of SLAMF6 in NKT cell development. Other SLAMF members were also found to regulate NKT cell development in a SAP dependent and independent manner [104].

It has also been determined that SLAMF6 acts as a SAP-dependent on and off switch for stable T-B cell interactions [79]. In the absence of SAP, SLAMF6 recruited the negative regulator SHP-1 at the T-B cell synapse limiting adhesion. Interestingly, when both SAP and SLAMF6 were removed ( $SAP^{-/-}SLAMF6^{-/-}$ ), both  $CD4^{+}$  T cell function as well as NKT cell differentiation was restored. Therefore, SLAMF6 also serves an important role in sending positive and negative signals depending on the competition between SAP and SHP-1 binding, which in turn regulates T cell help and NKT cell development.

### **3.6 SLAMF6 as a susceptibility gene for Systemic Lupus**

#### **Erythematosus**

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that mainly affects females and is characterized by the production of autoantibodies against self antigens [105]. Generation of these antibodies results in clinical manifestations including arthritis, kidney damage, skin disease and blood cell abnormalities. Both genetic and environmental factors are known to contribute to disease manifestation [105, 106].

Analysis of the SLAM/CD2 gene cluster revealed extensive polymorphisms, and among 35 inbred mouse strains two stable haplotypes were identified [60, 107]. Haplotype 1 is found in C57BL/6J (B6) and related strains while haplotype 2 is found in autoimmune-prone mouse strains, i.e. NZW[60]. It appeared that autoimmunity was induced only when the haplotype 2 SLAM locus was expressed in B6 background [60]. Sle1 was

identified on murine chromosome 1 as a cause of loss of tolerance and autoantibody production. Fine mapping of the *Sle1* locus identified 4 loci contributing to the disease manifestation, one of which was the most potent: *Sle1b* [60]. The congenic B6.Sle1b mouse strain presented with spontaneous autoimmunity and production of antinuclear antibodies (ANAs), indicating a failure to maintain central and peripheral tolerance [60, 108, 109]. Among the SLAMF genes, SLAMF6 was the strongest candidate for lupus due to the expression of alternatively spliced variants: Ly108-1 and Ly108-2. Of the two, Ly108-1 had a greater tendency to be phosphorylated at the cytoplasmic tail for SAP, Fyn recruitment and signaling than Ly108-2 isoform [110]. Kumar et al. and colleagues demonstrated that the Ly108-1 isoform was more abundant in B6.Sle1b mice compared to B6 mice, which was found to sensitize immature B cells to deletion and RAG re-expression [111]. This study provided the idea that SLAMF6 serves as a regulatory checkpoint for self-reactive B cells to protect from autoimmunity.

A later study identified a new isoform of Ly108 present in haplotype 1 but not haplotype 2: Ly108-H1 [61]. This isoform was found to regulate SLE in a CD4<sup>+</sup> T cell dependent manner and expression of this isoform in lupus-prone mice significantly suppressed autoimmunity. Deletion of SLAMF6 in Chr1b also disrupted the autoimmune phenotype. In addition, autoimmunity in B6.Sle1b mice correlated with expansion of an osteopontin-expressing TFH cell subset, which was suppressed when the Ly108-H1 isoform was expressed in these mice [112]. Overall, these studies pointed to the importance of alternative splicing in pathogenesis of SLE and that both B and T cell tolerance is compromised.

## 4 SLAMF6 in Hematological Malignancies

Hematologic malignancies are cancers that affect the blood, lymphatic system and the bone marrow. Malignant cells arise from blood cells of common lymphoid and myeloid progenitor origin and are categorized under three main subsets: leukemias (acute and chronic), lymphomas and myelomas [113]. Chromosomal translocations are a common cause of these diseases unlike solid tumors, and thus are commonly used as diagnostic factors.

As SLAMF members are expressed on a variety of normal immune cells that form the tumor microenvironment as well as cells that become malignant, it is plausible to hypothesize that SLAMFs may play a role in all these complex interactions. In fact, in recent years many investigators have demonstrated relevant roles for SLAMFs, SLAMF6 in chronic lymphocytic leukemia and SLAMF7 in multiple myeloma (MM), of which SLAMF6 will be discussed in further detail.

### 4.1 The Pathogenesis of Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) is the most common leukemia in western countries accounting for 40% of all leukemias. It is a malignancy of mature CD5<sup>+</sup> B cells that accumulate in the blood, bone marrow and secondary lymphoid organs [114]. Phenotypically they are similar to antigen-experienced B cells expressing CD19, CD5, CD23, CD25, CD69 and CD71, and the memory B cell marker CD27 [115-117]. CLL is broadly divided into 2 subsets depending on the immunoglobulin (Ig) heavy chain mutation status (IGHV). CLL B cells with unmutated IGHV (U-CLL) derive from mature

CD5<sup>+</sup> B cells, whereas CLL cells with mutated IGHV (M-CLL) derive from CD5<sup>+</sup>CD27<sup>+</sup> post-germinal center B cell subsets [118].

CLL patients can have varying clinical outcomes depending on the aggressiveness of the disease. While some patients are stable and are only observed over time without any need for treatment, some experience more aggressive disease and require immediate treatment. Multiple factors play role in determining the course of the disease. Mutational status of CLL B cells is an important prognostic factor and patients with U-CLL show a more aggressive disease and shorter survival time compared to M-CLL patients [119, 120]. Chromosomal alterations are another parameter for disease outcome. Patients with 13q deletions that include the miRNAs miR15a and miR16-1 are usually associated with favorable disease outcome [121]. Mouse models with deletion of the miR15a and miR16-1 locus mimic many features of human CLL [122]. Chromosomal deletion of 17p and 11q harbor the p53 and ataxia telangiectasia mutated (ATM) genes and are associated with poor disease outcome [121, 123]. Trisomy 12 is found in ~ 15% of CLL patients and signifies an intermediate prognosis. In addition to chromosomal aberrations, high expression of CD38 and ZAP70 in CLL B cells is associated with a poor outcome and shorter time to treatment [119, 124, 125]. Besides these clinical markers that are associated with disease outcome of CLL patient, studies using the application of whole exome sequencing have identified recurrent somatic mutations that are involved in DNA damage, mRNA processing, WNT and Notch signaling and chromatin modifications that can affect B cell signal transductions [126-129].

#### 4.1.1 CLL Microenvironment

Cells of the immune system coevolve with the tumor and provide the tumor a friendly microenvironment for survival of CLL cells. Culturing CLL B cells *in vitro* after isolation



from peripheral blood mononuclear cells (PBMCs) leads to the induction of spontaneous apoptosis indicating that CLL B cells are highly dependent on the microenvironment for survival signals [130, 131]. Only when cultured with bone marrow stromal cells, CLL B cells survived *in vitro*. This suggests that the tumor microenvironment *in vivo* has a profound effect on survival and expansion of CLL leukemic cells [114]. Some of the key interactions between CLL B cells and the tumor microenvironment are highlighted in **Figure 6**.

One of the CLL supporting cells is the 'nurse like cells' (NLCs), which are of monocyte origin [132]. These cells were found to differentiate *in vitro* from PBMCs of CLL patients and secrete chemokines such as CXCL12 and CXCL13 for their survival *in vitro* [132, 133]. These chemokines induce chemotaxis for migration of CLL cells in and out of secondary lymphoid organs *in vivo*. CXCL12 mediated signaling is increased by CD38 expressed on CLL cells further promoting survival [134, 135]. NLCs also activate the B cell receptor signaling and NF- $\kappa$ B pathway for survival [136].

The B cell receptor (BCR) signaling pathway is a key survival factor for CLL [137]. The BCR is composed of an antigen-specific surface membrane Ig (smlg) along with Ig $\alpha$ /Ig $\beta$  heterodimers. Engagement of the BCR triggers phosphorylation of Ig $\alpha$ /Ig $\beta$  and recruitment of Lyn, which in turn activates kinases SYK, BTK and PI3K. This leads to the activation of downstream signaling cascades including phospholipase C gamma 2 (PLC $\gamma$ 2), calcium signaling, NF- $\kappa$ B and mitogen-activated protein kinase (MAPK) pathways. These signaling events promote survival and proliferation of B cells [138]. IGHV mutation status of CLL cells determines the responsiveness of BCR engagement. While CLL B cells from U-CLL patients are more responsive to BCR stimulation and mostly recognize autoantigens [139, 140], M-CLL cells show constitutive phosphorylation of ERK kinase along with reduced surface BCR leading to an "anergic"

phenotype [141, 142]. In addition to activated BCR signaling, ligation of CD40/CD40L on malignant B cells and T cells also promote survival of CLL cells [143].

Another hallmark of CLL is dysfunctional T cells in the tumor microenvironment. Although there is an overall expansion in the T cell compartment, the normal CD4/CD8 ratio appears to be inverted due to differential sensitivity of CD4<sup>+</sup> and CD8<sup>+</sup> T cells to Fas/Fas Ligand induced cell death [144-148]. Within CD4 T cells, frequency of regulatory T cells (Treg) is increased further supporting leukemic expansion [149-151]. CD4<sup>+</sup> and CD8<sup>+</sup> T cells show many defects in CLL including T cell exhaustion, inability to form immune synapses and impaired cytotoxic function [152-154]. Exhausted CD8 T cell state correlates with increased expression of exhaustion markers such as programmed death-1 (PD-1), CD160, SLAMF4 and KLRG1 [152, 155-157]. Several studies hypothesized one factor for exhaustion may be cytomegalovirus (CMV) infection that influences and expands the CMV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets in healthy individuals and is also expanded in CMV-seropositive CLL patients [158-160]. However, within the exhausted T cell pool, CMV-specific T cells showed reduced expression of exhaustion markers as well as retained cytotoxic capacity and cytokine production compared to other exhausted T cells [161, 162]. These data indicated that there might be other factors influencing changes in the T cell compartment in CLL.

PD-1/PD-L1 is a major pathway contributing to the known T cell defects in CLL [163]. PD-L1 is overexpressed on CLL cells and myeloid derived suppressor cells (MDSCs), which further up-regulates PD-1 on T cells [164, 165]. Within CD4 and CD8 T cell populations, naïve T cells are reduced while effector memory CD4<sup>+</sup> T cells and terminally differentiated CD8<sup>+</sup> T cells are increased, which clinically corresponds with disease aggressiveness [163]. Up-regulated PD-1 inhibits IFN $\gamma$  secretion skewing the

immune responses to a dysregulated T<sub>H</sub>2 response [163]. These T cell defects are recapitulated in the TCL1 transgenic (E $\mu$ -TCL1) mouse model of CLL [166-168], allowing a useful platform to study and understand the contribution of PD1/PD-L1 pathway in CLL pathogenesis and how this pathway can be targeted therapeutically.

#### 4.1.2 Therapeutic options in CLL

Allogeneic hematopoietic stem cell transplantation (HSCT) represents one of the oldest treatments for hematological malignancies which set the foundation for the development of cancer immunotherapy [169]. Performed for the first time in 1968, high doses of radiation and chemotherapy were given to the patient that wiped out the entire immune system followed by donor HSC transplantation for repopulation of the hematopoietic system. Extensive studies over the years provided great insight into the efficiency of the ability of donor immune cells to eliminate recipient tumor cells. This is known as 'graft versus leukemia' (GVL) effect. GVL potency was further appreciated by the finding that post- HSCT, donor lymphocyte infusions (DLI) induced remarkable responses and remissions without radiation or chemotherapy, in leukemias [170, 171].

Targeting BCR signaling using small inhibitor molecules has dramatically improved treatment options for CLL patients [137]. The BTK inhibitor, ibrutinib (PCI-32765) binds irreversibly to a cysteine residue (Cys-481) in the BTK kinase domain and inhibits its phosphorylation and enzymatic activity [172]. Ibrutinib inhibited proliferation and stromal cell contact of CLL cells and reduced their viability *in vitro* [173]. Ibrutinib also prevented tissue homing in response to chemokines CXCL12 and CXCL13 *in vitro* and in a mouse model of CLL [174]. This inhibition of tissue homing chemokines directly correlated with

a transient lymphocytosis in CLL patients undergoing ibrutinib treatment, which allowed CLL cells to move from secondary lymphoid organs into the circulation and induced cell death [175]. Because ibrutinib not only binds to BTK in CLL B cells but also to ITK in T cells, its immunomodulatory role was also investigated in CLL patients [176]. In patients treated with ibrutinib, the increased T cell numbers normalized and production of inflammatory cytokines were reduced, and the T cell repertoire diversity increased [177, 178]. Ibrutinib also reduced Treg numbers [179]. Expression of PD-1 and PD-L1 upon ibrutinib treatment markedly decreased, improving activated effector T cell functions [180, 181].

While ibrutinib targeted CLL B and T cells, a second-generation highly selective BTK inhibitor, acalabrutinib, was produced [182]. Pharmacodynamics and proteomic analysis appeared to be similar on leukemic cells compared to ibrutinib, while off target effects on T cells was more pronounced using ibrutinib than acalabrutinib [183, 184].

Rituximab, a monoclonal antibody against the B cell surface antigen CD20, is widely used in the treatment of B cell malignancies including CLL [185-187]. The primary mode of action of rituximab includes ADCC and complement dependent cytotoxicity (CDC) as well as direct anti-proliferative and pro-apoptotic effects [188, 189]. While major advances were brought by rituximab, relapse and resistance to treatment are eventually seen in patients. One potential reason is the removal of bound CD20 complexes from the surface of CLL cells by trogocytosis [190, 191]. Administering rituximab together with ibrutinib led to better responses, but follow up studies identified that ibrutinib interferes with the effect of rituximab by downregulating CD20 on the cell surface of CLL cells [192, 193]. More efficient combinations with rituximab may be required for better and durable effects of this immunotherapy agent.

Lenalidomide is an immunomodulatory agent that affects the tumor microenvironment and the immune system. In particular, it corrects CLL B cell - T cell immunological synapse formation and down-regulates PD-1 on T cells [153, 164, 194]. Lenalidomide normalizes total T cell and Treg numbers, similar to ibrutinib, *in vivo* [195]. Combining lenalidomide with  $\alpha$ CD20 improved ADCC activity of NK cells *in vitro*, and this combination also demonstrated efficacy in clinical trials [196, 197]. Other small inhibitor molecules targeting BCR signaling, monoclonal antibodies and immunomodulatory drugs used in treatment of CLL patients are discussed in detail elsewhere [137, 198].

## 4.2 SLAMF Receptors in CLL

The importance of SLAMF receptors in regulating innate and adaptive immune responses makes them relevant candidates in context of various diseases including chronic lymphocytic leukemia. SLAMF receptors, particularly SLAMF6 is expressed on both human and mouse CLL cells [199].

SLAMF6 expression is high on normal T and B cells and studies have already indicated its importance in B-T cell signaling. Therefore it was plausible to hypothesize that monoclonal antibodies targeting SLAMF6 may be of therapeutic interest in CLL [199]. An adoptive transfer model of an aggressive TCL1 clone, TCL1-192 [200], into SCID (severe combined immune deficiency) mice, which lack T and B cells, was injected with anti-SLAMF6 upon leukemic. This led to significantly reduced leukemic burden by inducing antibody dependent cellular cytotoxicity (ADCC) and reduced proximal B cell receptor signaling [199]. Interestingly, the antibody was unable induce ADCC in

peritoneal cavity (PerC) of mice, due to possibly different microenvironments and signaling compared to blood or spleen [199, 201]. One such finding was the elevated reactive oxygen species (ROS) production in PerC CLL B and normal B1a B cells compared to the cells residing in spleen [201]. While inhibition of ROS limited leukemic expansion in this niche, the finding that ROS is reduced upon BTK inhibitor, ibrutinib and leukemic infiltration to the blood, prompted us to do a combination therapy. By combining anti-SLAMF6 with ibrutinib, leukemic cells were pushed out of this niche into the circulation making them targetable by the antibody. This led to an overall greater reduction in leukemic burden than either regimen alone. Exploration of SLAMF6 as a therapeutic target would be of great interest in CLL and other B cell malignancies.

## 5 Conclusions

SLAMF6 receptor plays vital roles in maintaining a balanced immune response and its interrupted functions are associated with various diseases. Ability of SLAMF6 receptors, as well as other SLAMFs, to play activating and inhibitory roles depending on the signals they receive or the cell-cell interactions they encounter makes them an important target when thinking about designing therapeutics. Monoclonal antibodies that can block or engage SLAMF6-SLAMF6 interactions are being targeted in diseases such as CLL. Further understanding the fundamentals on how SLAMF6 plays a role in the bigger picture will make them one step closer to being targets in many more diseases.

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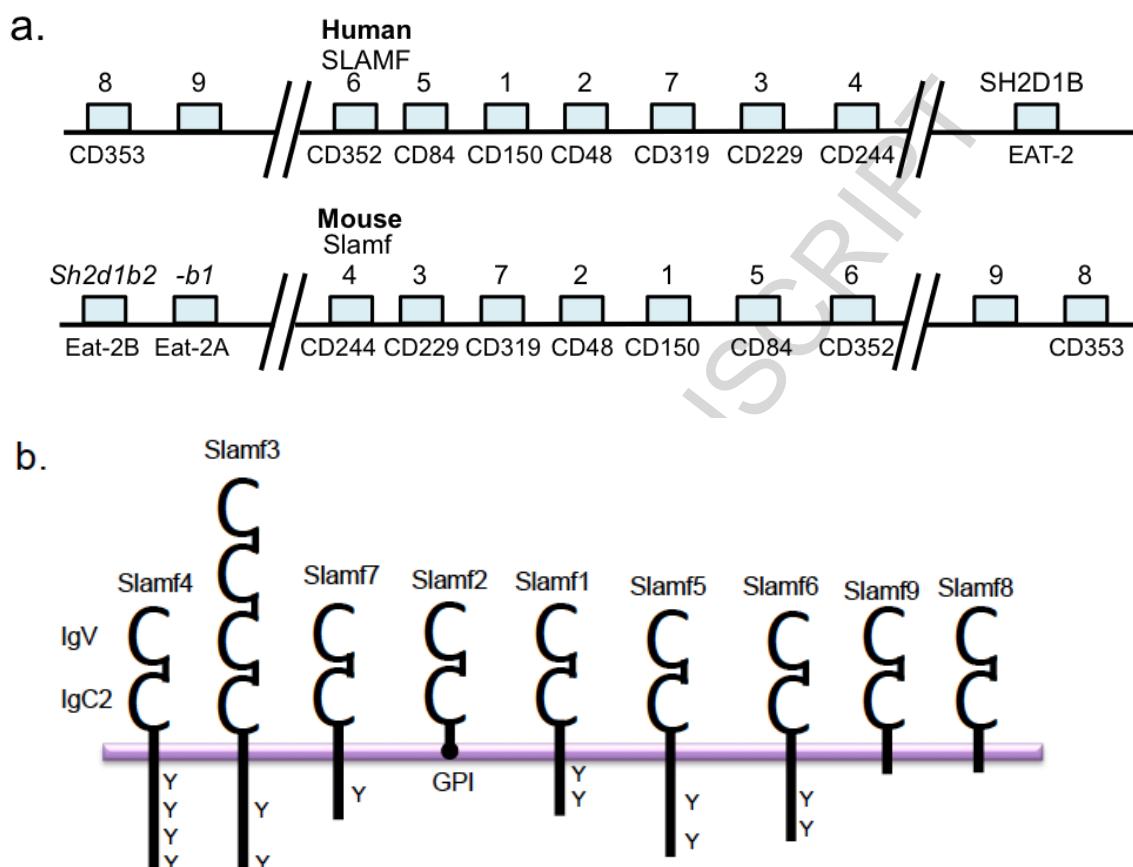


Figure 2

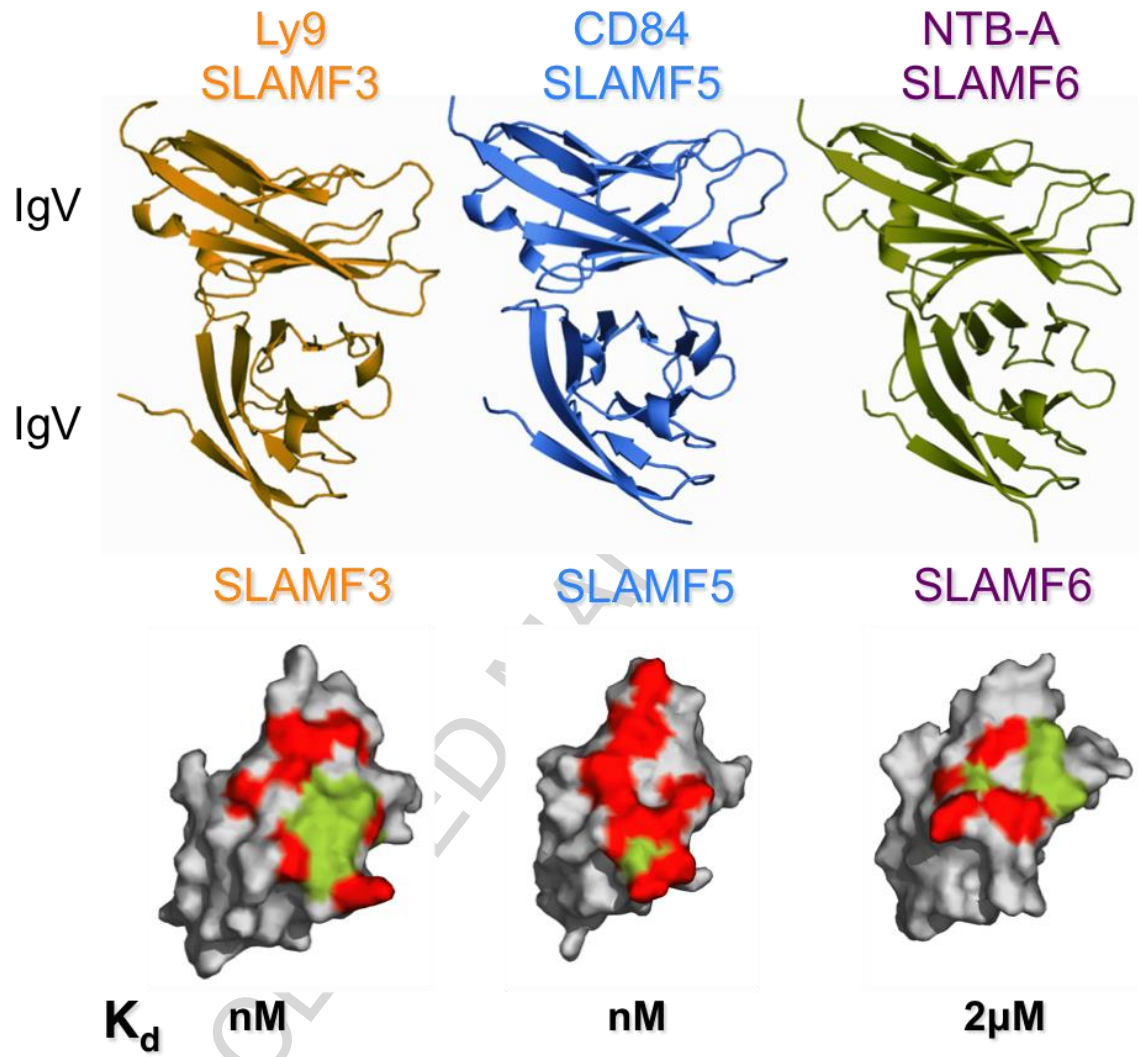
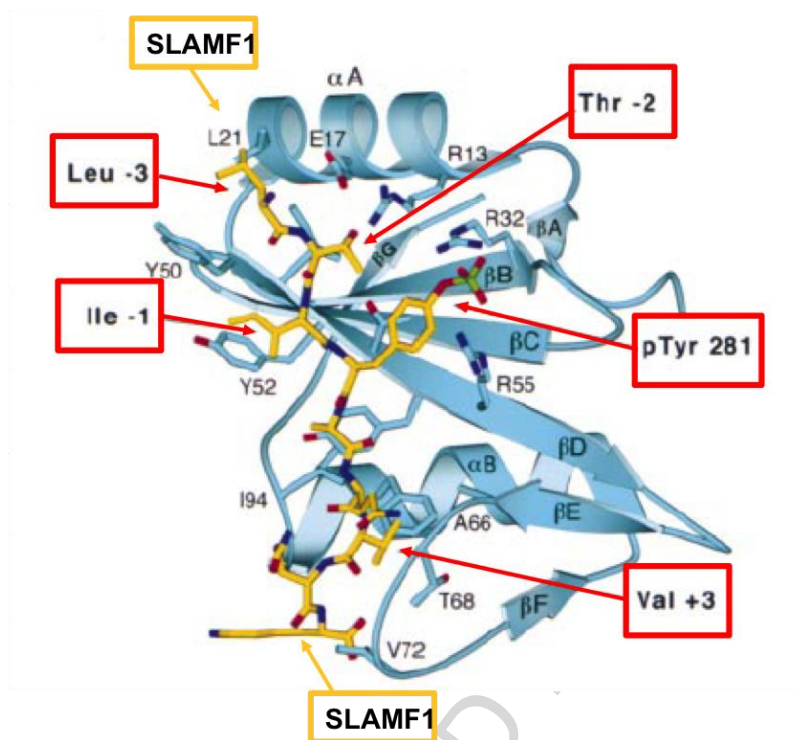


Figure 3



### Figure 4

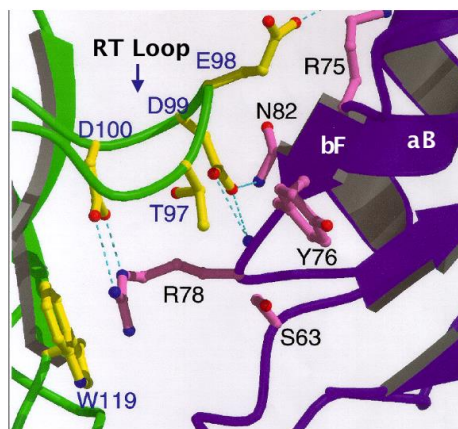
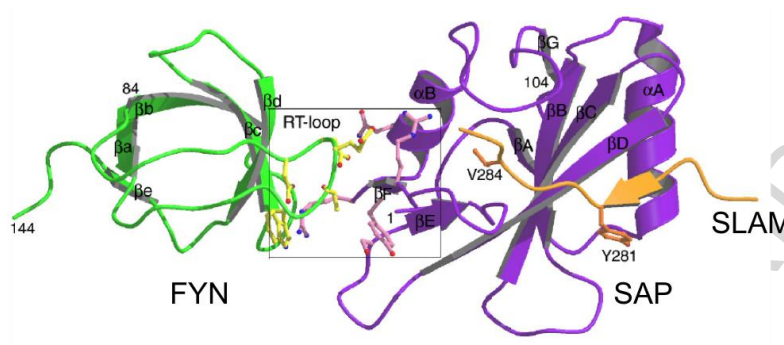


Figure 5

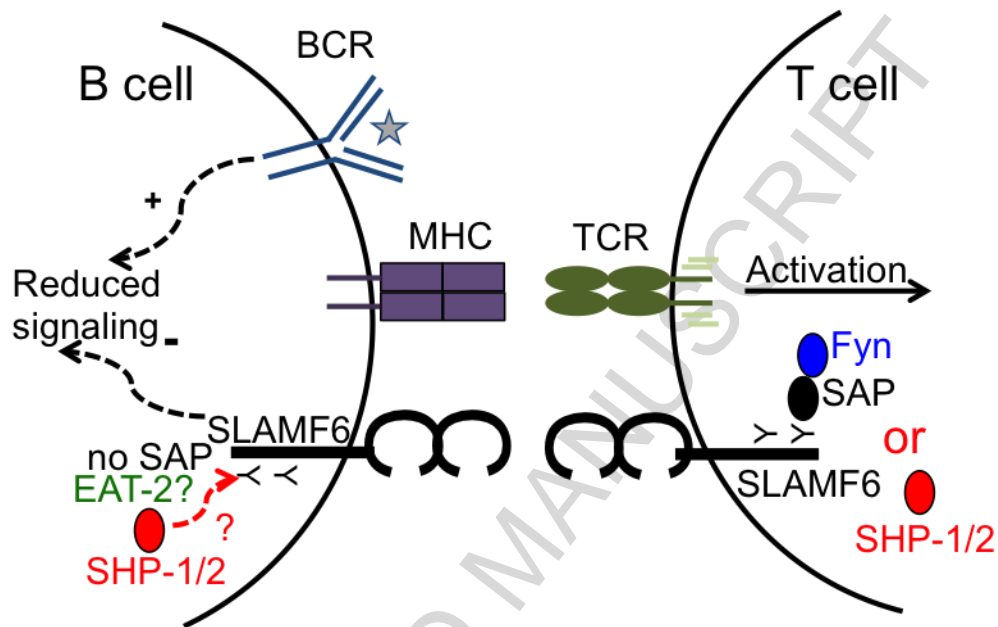
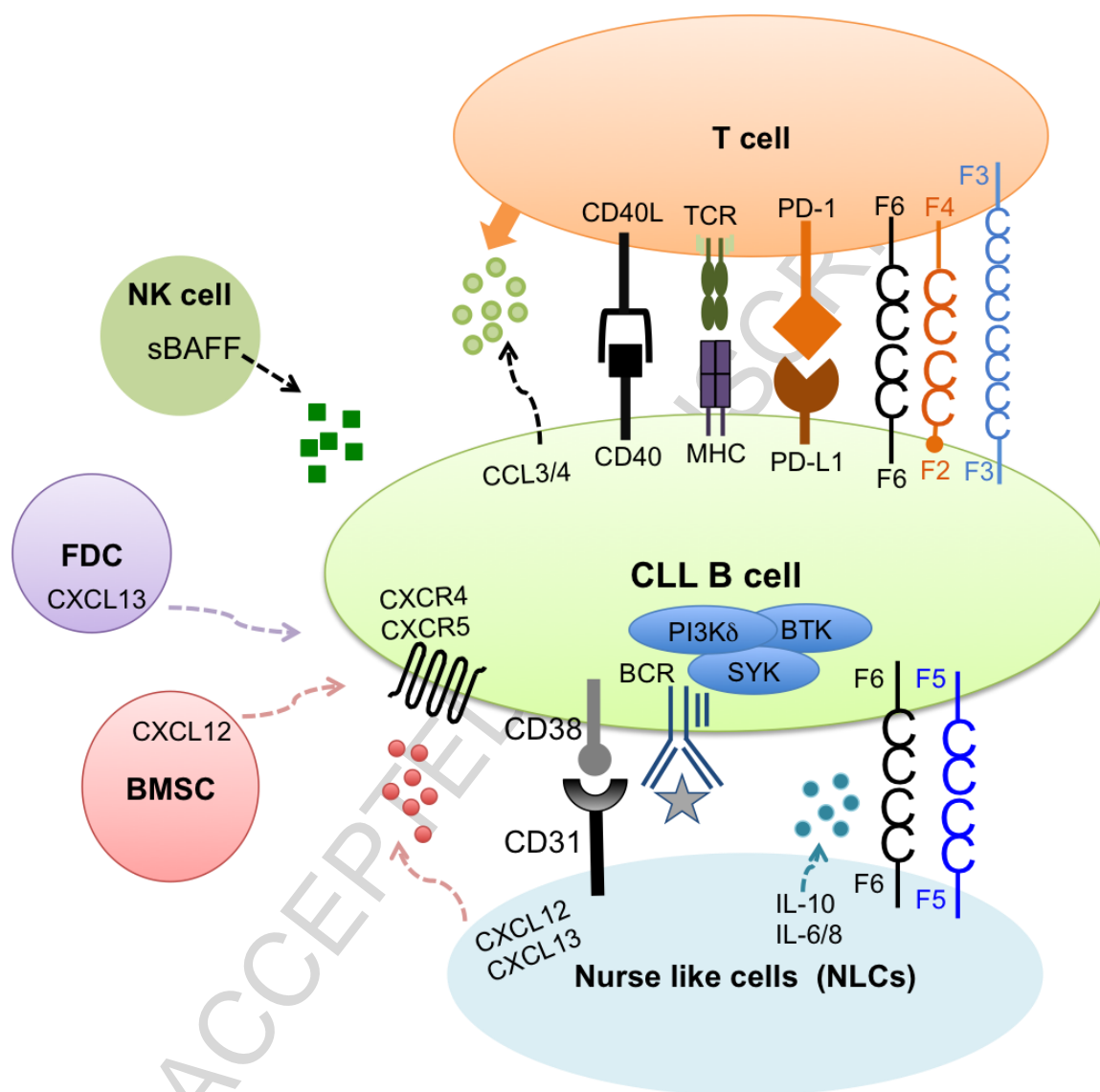


Figure 6



**Figure 1. Signaling lymphocyte activation molecule family (SLAMF) of genes and protein.** **a.** Organizational overview of SLAMF gene cluster on chromosome 1 in human and mouse. **b.** SLAMF members consist of an IgV/IgC2 ectodomain, which is duplicated in SLAMF3. While SLAMF2 and SLAMF4 bind each other, other SLAMF receptors are homophilic. Six members of the family contain varying lengths of cytoplasmic tail with ITSM motifs (Y) that can recruit and bind the adaptors SAP and/or EAT-2.

**Figure 2. Homophilic engagement of SLAMF3, SLAMF5 and SLAMF6 occurs via interactions of the IgV domains.** Specificity of homophilic binding is determined by different surface characteristics. All three SLAMF receptors show different binding affinities. Green = hydrophobic, red= hydrophilic amino acids

**Figure 3. Ribbon diagram showing SAP/SLAMF1 pY281 complex.**

The bound SLAMF1 phosphopeptide is shown in a stick representation (yellow). Selected SAP residues that form the binding site are shown in blue. SLAMF1 residues N-terminal to pY281 make additional interactions with SAP at pY -3 and pY -1 (positions relative to pTyr281)[34, 35].

**Figure 4. SAP couples Fyn to SLAMF receptors.** SAP binds the Fyn SH3 domain through a non-canonical surface interaction (zoomed area). SLAM peptide binds SAP in a 3-pronged mode by via the b-strand of N-terminal, Tyr 281 and Val 284.

**Figure 5. SLAMF6 localizes at the immune synapse.** Ligation of SLAMF6 on a T cell recruits binding of SAP to the ITSM on the cytoplasmic tail. SAP recruits Fyn and



induces activation. SAP has the highest affinity for cytoplasmic tail of SLAMs and blocks binding of SHP-1/2. In the absence of SAP, SHP-1/2 binds to cytoplasmic tail of SLAMF6 and induces negative signaling on T cells [79]. B cells do not express SAP. Whether EAT-2 is expressed or it blocks recruitment of SHP-1/2 is not known. However, ligation of SLAMF6 with an antibody in B cells appear to induce negative signals, which may be due to binding of SHP-1/2.

**Figure 6. The CLL Microenvironment.** Contact between CLL cells and nurse like cells (NLCs) is established by chemokine receptors and adhesion molecules expressed on CLL cells and ligands on NLCs. The CD38-CD31 axis promotes CLL survival. CXCR4/CXCL12 chemokine gradient allows shuffling of CLL cells between circulation and secondary lymphoid organs to receive survival and proliferation signals. SLAMF5 and SLAMF6 are expressed on the surface of NLCs (Yigit, unpublished data). The relevant contribution of these receptors to CLL survival requires further investigation. T cells are another major contributor to CLL survival. Secretion of chemokines CCL3/4 by CLL cells attracts T cells nearby. CD40/CD40L interaction promotes survival and PD-1/PD-L1 pathway favors immune evasion of CLL cells. NK cells, bone marrow stromal cells (BMSCs) and follicular dendritic cells (FDCs) also contribute to CLL survival.

### Highlights

- SLAMF6 is a cell surface receptor, expressed only on hematopoietic cells
- SLAMF6 receptor can positively or negatively regulate immune responses
- SLAMF6 have been implicated in autoimmunity and cancer and exploiting this receptor using monoclonal antibodies can help improve current treatment in various diseases